VOL. XV, PP. 213-234 NOVEMBER 20, 1902

PROCEEDINGS

OF THE

BIOLOGICAL SOCIETY OF WASHINGTON

OBSERVATIONS ON THE CONDITIONS OF LIGHT PRODUCTION IN LUMINOUS BACTERIA.

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Introduction.

Although the phenomenon of the production of light by organic or organized matter was known in the days of Aristotle, comparatively little is known concerning the actual conditions of luminescence. The number of organisms or organic bodies said to possess the so-called phosphorescent* properties is a large and varied one. Both animal and plant forms, simple and complex types of life, are to be found in the category of light-producing organisms.

In view of the comparative ease of culture and simplicity of form, the photogenic bacteria constitute perhaps the best material for the study of luminescence in living forms—a phenomenon, the essential conditions of which are probably the same in all forms of life. The study of the conditions of luminescence was undertaken at the suggestion of Prof. Dr. W. Pfeffer and was carried out in the botanical laboratories of Leipzig and Basel. It is a pleasant duty to here give expression to my appreciation of the friendly counsels of Professors W. Pfeffer and A. F. W. Schimper during the course of my experiments.

*The term phosphorescence as applied to the light produced by luminous organisms is inappropriate. It should only be applied to light emitted in the dark by bodies which have been previously illuminated. The light emitted by all known luminescent forms of life is entirely independent of previous illumination.

Literature.

It is only a quarter of a century since Pflüger discovered the first luminous bacterium—*Micrococcus phosphoreus*, Cohn.* Previous to this discovery, Pflüger had expressed the belief that the (so-called) phosphorescence phenomena would prove to be intimately connected with the respiratory processes. He obtained confirmation of this belief when by experiment he showed the need of free oxygen for the production of light by the newly discovered bacterium. Since light production then seemed bound up with respiration, Pflüger asserted that luminescence was a vital phenomenon—that it was inseparably bound up with life. Those who had studied luminosity in animals before Pflüger's discovery of luminous bacteria had arrived at the conclusion that it was the protoplasm which was luminous—that the luminous matter was "lebendiges Eiweiss." Pflüger's experiments apparently confirmed this position.

In 1880, Radziszewski attacked the question from the chemical standpoint. It had previously been known that certain substances when raised to a certain temperature could be made to emit light. Radziszewski discovered a large number of additional non-living organic compounds which could be made to give out light. He also found that in many cases a high temperature was not required and that with lophin no higher temperature than 10° was needed. Further he ascertained that the conditions of luminescence were a markedly alkaline reaction and a slow oxidation. Organic and inorganic bases might be employed to produce the alkaline reaction. The light produced by these substances has a spectrum very closely resembling that of the photogenic forms—giving a continuous band between D and G, with the brightest part between E and F. In view of the resemblance of the spectra of the light produced by these chemicals and by luminous organisms, Radziszewski is of the opinion that the light of luminous organisms is due to the presence of certain of the photogenic substances which he discovered. While Radziszewski differs from Pflüger in holding luminescence not to be a vital phenomenon, yet both agree that oxidation plays a very important rôle in luminescence. Dubois records a symbiotic relation between a marine mollusk and a species of bacterium. In this case Dubois asserts that the animal excretes a substance "Luciferin" which through action of the bacterium is caused to emit light.

Beijerinck, who has studied a number of forms, finds that light ceases with the death of the bacteria and that the light intensity may be diminished or increased by varying the nature of the nutrient media. His conclusion concerning luminescence is, that it is a vital process—that it is due to the liberation of radiant energy by the oxidation of peptone at the moment of its conversion into living protoplasm.

The observations of B. Fischer, Forster, Lehmann and Tolhausen of the production of light at and below 0° seem hardly to agree with the

*Since 1875 there has been as many as twenty-five species of luminous bacteria recorded from widely separated parts of the world. In all probability, many of these species names will prove to be synonyms, or at least varieties.

theory of the vital nature of luminescence. However, these observers hold that inasmuch as no luminous substance has ever been isolated from photobacteria, luminescence must be inseparable from life. Ludwig, and with him Dubois, is of the opinion that the light is produced by some specific substance, similar to those discovered by Radziszewski.

As the matter rests now, there is almost as much evidence for the "luminous substance" theory as for the intracellular vital theory; the latter having, however, slightly the better of the argument. In order to come a little nearer to the cause of light production, I decided to examine more minutely into the nutrition of the photobacteria and the effect of various external agents on the light production.

Material.

Most of the succeeding observations and experiments were made with *Bacillus phosphorescens*, B. Fischer (*Photobacterium indicum*, Beij.) and *Microspira luminosa*, (Beij.) Mig. (*Ph. luminosum* Beij.). Some experiments were also made with *Bacterium phosphorescens*, B. Fischer, (*Ph. phosphorescens*, Beij.). Cultures of these species were obtained from Kral's laboratory in Prague. The culture of Microspira luminosa thus obtained emitted a weak light. Strongly luminous cultures of this species were obtained, however, through the kindness of Prof. Dr. Beijerinck, of Delft.

The morphologic characters of the above mentioned species are quite fully set forth in the papers of Beijerinck and in Migula's "System der Bakterien." It may simply be noted here that the Bacillus and Microspira are motile and liquefy gelatine, while the Bacterium is non-motile and does not liquefy gelatine. When not otherwise indicated the results recorded will refer to *Bacillus phosphorescens.*

General Methods of Culture.

For most of the experimental work a liquid culture medium was found best, but control experiments were frequently made with solid culture media. About 500 grams of fresh fish were extracted over a water bath with two litres of water. Herring, pike and carp yielded good extracts, but that obtained from a couple of species of flounder was decidedly less favorable to both growth and light production. To the filtered fish extract the following ingredients were added:

Peptone	1.0%
Asparagin	.5%
Glycerol	2.0%
Na Cl	2.0%
Mg Cl_2	1.0%

The liquid thus obtained was made weakly alkaline with Na OH, and constitutes what will later be designated as normal fish bouillon. Appropriate solid media were obtained by adding to this bouillon either 1% of good agar or 6 to 8% of best grade gelatine.

As containers for the bouillon, Erlenmeyer flasks of ca. 100 c. c. capacity were employed. From 10 to 20 c. c. of the bouillon was introduced into each flask. The broad base of the flask at once insured

stability of the cultures and permitted access of free oxygen to all parts of the media.

Relations to Acids and Bases.

In his study of *Bacterium phosphorescens*, Beijerinck came to the conclusion that certain acids, e. g. lactic, malic, glyceric and aspartic, accelerated light production, while others, such as formic, acetic, propionic and butyric decreased light emission. Still others, e. g. citric, mucic. oxalic and glycolic, appeared to be without effect on the bacterium. Beijerinck further observed that certain of the salts of these acids reacted toward the bacteria much as did the free acid. That free acids should in all cases prove injurious, or even that they should all be beneficial, would not be particularly surprising, This variation in the action of the acids was, however, difficult to understand.

A quantity of normal fish bouillon, agar, and gelatine were made weakly acid with HCl, HNO_3, H_3PO_4 and CH_3COOH respectively. These media were then inoculated with *Bacillus phosphorescens* and *Bacterium phosphorescens;* but no growth ever appeared. Since the acid might perhaps inhibit initial, but not later growth, and not the light production, luminous cultures were obtained in normal media and the acids then added.

To a stab gelatine culture of *Bacterium phosphorescens*, 4 drops of decinormal HCl were added with a pipette. The acid was dropped directly *on* the bacterial growth. The light was instantly extinguished. During the 6 hours following treatment no light was emitted, but after 24 hours a faint light was visible in the culture. This experiment was repeated a number of times and each time with the same result. Evidently the acid was injurious to light production, but not for the life of the organism.

In a second series of experiments, cultures in normal fish bouillon were employed. To such cultures, which were strongly luminous, 4 drops of decinormal HCl were added. No effect on light emission was observed. Examination showed that the amount of HCl added had not been sufficient to give the medium an acid reaction. Decinormal HCl was then added to another lightning bouillion culture until the light emission ceased. The culture fluid was then found to be slightly acid to litmus paper.

Normal, double normal, and fairly concentrated HCl were in turn added to a series of light-emitting bouillon cultures until the light disappeared. In each case the media at the end of the experiment were slightly acid to litmus. Naturally the more concentrated the acid the less was required to cause cessation of light production. In no case, however, did the light disappear until the media became slightly acid. The experiments were repeated in bouillon cultures containing litmus. In all cases light disappeared as soon as the medium turned faint red, i. e., was acid, and not before.

One may interpret the difference between the results obtained with solid and fluid cultures by the fact, that with the solid media the acid acted at once on all of the bacteria, while in fluid culture only some of

the bacteria were subject to the action of the acid before its neutralization. Immediately the media became acid, i. e., the moment all the bacteria were subjected to the action of the acid, the light instantly disappeared.

Numerous experiments with nitric, sulphuric, orthophosphoric, formic, acetic, lactic, succinic, malic, tartaric, oxalic and citric acids, gave results essentially the same as those obtained with hydrochloric acid. At the moment the media turned just weakly acid, the light emission at once ceased. Naturally, in proportion as the normal acid was weak, or the acid dilute, so was the actual quantity of acid solution required to give an acid reaction to the medium and destroy light, the larger. The end result of a dark culture and slightly acid reaction of the medium was the same in all cases.

A few experiments were made to learn the effect of the acid salts. The dihydric phosphates of sodium and potassium—NaH_2PO_4 and KH_2PO_4—were employed for this purpose. Quite large quantities of the solutions of these salts were needed to render the culture media acid. In each case, however, as soon as the medium became slightly acid, the culture became at once dark.

In cultures thus treated with acids the light never returned. In most cases, even when the culture was made weakly alkaline within five minutes of the acid treatment, light did not again appear in the culture. In cultures which had been made alkaline after acidification with the acid phosphates, light was again emitted within 12 hours of the addition of the alkali.

A few experiments to learn the effect of excess of Na OH and KOH in the media were also tried. Growth only occurs in media which turns red litmus light blue. If 2 to 4 drops of decinormal KOH or NaOH be added to a good luminous bouillon culture light production ceases instantly, and subsequent reduction of excessive alkalinity never permits any return of light. Inoculations made from such cultures do not take, showing the bacteria to have been killed and not simply rendered inactive, as is the case when light is destroyed by acids.

The experiments here recorded for *Bacterium phosphorescens* were repeated with *Bacillus phosphorescens* and *Microspira luminosa*. Like results were obtained with both.

It may be well to briefly note here the methods employed for the introduction of reagents into the cultures. In the preliminary experiments the cotton plug was removed, the quantity of sterile reagent quickly introduced with a pipette and the cotton plug at once replaced. Although experience showed that there was rarely any bacterial contamination by this method, still there was the danger. Since the cultures were kept under observation for some days after treatment, a method of experimentation was devised which entirely precludes bacterial contamination during the course of the experiment.

Small glass tubes were taken, drawn out to form small capillary tubes and on one end of such a tube a very thin-walled bulb was blown. Care was taken to have the walls of the tube heavier than the bulb wall. A measured quantity of the desired reagent was introduced into the bulb

and the open end of the tube sealed. The tube was then shoved through the cotton plug so that the bulb was just a little distance above the culture fluid in the bottom of the flask. The tube and bulb with the contained reagent were found light enough to be held in place by the cotton plug. The arrangement is shown in Fig. 1.

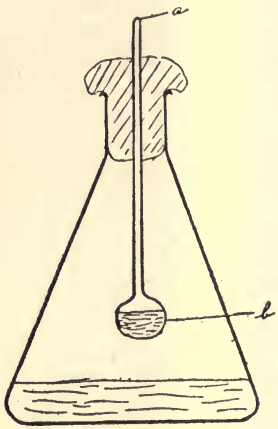

FIG. 1. Diagram of culture flask and bulb for introduction of sterile reagents.

The whole, containing culture media and reagent was sterilized in the usual way, the cotton plug slightly raised to permit the insertion of the needle and the media inoculated. When the culture is luminous and the reagent is to be applied, one presses on the end of the tube (a) and the bulb (b) is pressed against the bottom of the flask and shattered, bringing the reagent in direct contact with the bacteria. In this method, the only danger of contamination is that which is usually incidental to inoculation, and this experience shows to be extremely small.

The acids used in these experiments include mona-, di-, and tribasic members of both the inorganic and organic series. Since these representative acids all destroyed light emission and often the life of the organism, it seems probable that all acids would react in the same way. My results, then, are opposed to the conclusion of Beijerinck. I find all acids to be injurious to light production, lactic and malic (which Beijerinck distinctly labels photogenic) fully as much as citric and acetic (which he classes as indifferent and injurious respectively).

A consideration of Beijerinck's methods may explain his results. In the auxanogram method which he used, the reagent was dropped on a nutrient gelatine plate containing a rich bacterial growth. The acid diffused in radiating fashion from the point of contact, and as the diffusion circles widened, the reagent came in contact with the bacteria. Now the effect noted could not have been that of the free acid, since the moment the acid came in contact with the gelatine it would react with the contained alkali and form a salt. Consequently the effect noted must have been that of a probably neutral salt and not that of the free acid. The beneficial effect of the salts of certain acids noted by Beijerinck, I have been able to confirm. It is clear then that the error in Beijerinck's account, as far as acids are concerned, is not one of result but of interpretation of these results, since his experiments did not show the effect of the free acid, but rather of its salts.

The few experiments conducted with the hydrates of sodium and potassium show that while the photobacteria thrive in and, in fact, need a slightly alkaline nutrient medium, still the maximum point is rather sharply defined, and but a slight excess over that is even more fatal than an excess in the other direction.

Relations to Temperature.

Aside from some observations as to the maximum, minimum and optimum temperatures for light production, nothing has been recorded as to the relations of photobacteria to temperature. If the phenomenon of luminescence be primarily an irritabile function, we would expect to find evidence of this in the relations of the organisms to temperature. It must be borne in mind, however, that the term irritability rather expresses our ignorance than our knowledge of the phenomena usually classed under that designation. And this is especially true for the lower forms of life.

In the subjoined tables I give the earlier as well as my own records for the temperature minima, maxima and optima for both luminescence and growth in the three species I have examined.*

		Beijerinck	Migula	Fischer	Lehmann	Forster	Ludwig	McKenney
BACILLUS PHOSPHORESCENS								
Maximum	Growth							39°
	Light							30° (35°)
Optimum	Growth	Above 24°	20–30°					22–28°
	Light	30–35°	25–30°					22–28°
Minimum	Growth							15°
	Light							15°
MICROSPIRA LUMINOSA								
Maximum	Growth							30°
	Light	20°						22°
Optimum	Growth							15°
	Light	15°						15°
Minimum	Growth							10°
	Light							10°
BACTERIUM PHOSPHORESCENS								
Maximum	Growth					35–37°		38°
	Light		32°		39.5°	32°	38–39°	30°
Optimum	Growth							15–20°
	Light	10–15°	15–24°	5–10°	24°		15–20°	15–20°
Minimum	Growth							10°
	Light			0°	-12°	0°	10°	10°

*Here and wherever else in my paper temperature records are given they are according to the Centigrade scale.

It will be noticed that not only are the previous records fragmentary, but as the observers have multiplied there has been a diversity of result. As a result of my observations I am convinced that the optimum temperature for growth is the same or very nearly the same as that for luminescence.

Concerning the minimal temperature for luminescence the records are remarkably diverse, having a range of over twenty degrees. In all of my experiments the lowest temperature for luminescence *coincided* with that for growth. This does not mean, however, that the life becomes extinct below the minimal growth point. I have repeatedly kept cultures at 10°, at 0° and at -5° between 24 and 60 hours, and while there was never any growth at these temperatures, still when the cultures were subsequently placed at the optimal temperature a good growth and good luminescence resulted. The light, however, obtained in such cultures, particularly in those which had been kept below 0°, was especially brilliant, fully twice as strong as that of control cultures which had been kept at the optimum from the time of inoculation.

In neither of the three species was I able to observe light below 10° and I am of the opinion that the light which Lehmann observed in *Bacterium phosphorescens* at -12° must have been flourescence rather than true luminescence.

EFFECT OF TEMPERATURE CHANGE.

Having established the minimal, maximal and optimal temperatures, I next endeavored to determine the effect of change of temperature on light production. Good luminous cultures of Bacillus phosphorescens which had been grown at 26° were placed at 20°. Although these cultures were kept under continuous observation for 1 hour, no change was observed in the intensity of the light emitted. Again, luminous cultures were taken from 26° and placed at 15°. In from 12 to 15 minutes light emission ceased, and did not again return in 24 hours. The cultures were then placed at 26° and in the course of 30 minutes they were again luminous. In these changes of temperature the change as ascertained by a thermometer kept in the culture was comparatively slow and gradual.

Luminous cultures from 26° containing a thermometer, were plunged into water of a temperature of 5°. In from 1 to 2.5 minutes (a variation due probably to thickness of flask), the temperature of the culture had reached 15° and in 15 to 20 seconds more the culture had reached a temperature of 12°. The light intensity remained bright and even until between 14° and 15° was reached, when it instantly disappeared. Some of the cultures were kept between 10° and 12° for an hour and throughout this time no light was emitted. After a few minutes exposure at 12° some of the cultures were raised to a temperature of 15° in 10 seconds by plunging in hot water. Immediately this temperature had been obtained a weak light appeared, and when the culture had reached 25° the light was very brilliant. Of the cultures which had been kept between 10° and 12° for 24 hours some were slowly, others rapidly, raised

in temperature. In those in which temperature was gradually raised, the luminescence at once appeared as soon as the culture had reached 15°. In those in which temperature was rapidly raised by plunging in hot water, a temperature of 25° was obtained in 30 seconds, and with it strong luminescence.

The effect of changing from the optimum to a higher temperature was next studied. Cultures from 26° were gradually (in 15–30 minutes) raised to a temperature of 30°. Light continued with unabated brightness until 29.8° or 30.1° was reached and then the cultures immediately became dark.

Luminous cultures from 26° were plunged into water of 70°. In from 1 to 1.25 minutes the cultures were at 45° and the light instantly disappeared. Some of the cultures were slowly (15 to 20 minutes), others quickly (2 minutes) brought back to a temperature of 26°. Luminescence did not begin, however, as soon as the optimum had been reached. Usually it was from 10 to 12 hours after such treatment before the cultures were again luminous.

A number of experiments were also made in which old cultures that had ceased to emit light, and young cultures which were not yet luminous, had their temperatures raised and lowered, both gradually and rapidly. In no case, however, did any luminescence result, except in the very young cultures, which emitted light at the time in which untreated control cultures were also luminescent.

ACCOMMODATION.

In the preceding experiments it was found that for a very short period (1–2 minutes) it was possible to have luminescence as much as 15° above the normal maximum temperature for light production. Accordingly it seemed desirable to learn whether the bacteria would adapt themselves to life at higher temperatures and emit light. From the tables it will be observed that growth is possible for 9° above the normal maximal point for luminescence.

Normal fish bouillon (*a*) was then inoculated with *Bacillus phosphorescens* and placed at 35°. A good growth was soon noticed, but during two days no light was observed. From these cultures fresh inoculations were made into another lot (*b*) of media. A rapid growth occurred, but no light was produced. After 24 hours growth, fresh inoculations were made into a third lot (*c*) of media. In these cultures growth was luxuriant like the preceding, but no light appeared in 24 hours. Inoculations were again made to a fourth lot (*d*) of media. Growth was good in these cultures, but they still remained dark, and after 24 hours transfer was made to a fifth lot (*e*) of media. In these cultures not only was the growth good, but 12 hours after inoculation a weak light was observed. A little while before this (2 hours) a weak light was also noted in the *d* cultures, which were then 34 hours old. Transfers were successively made from the *e* set of cultures to sixth set, *f*, and from *f* to a seventh

set, *g*. All of the *f* and *g* cultures became luminous in from 12 to 18 hours from time of inoculation. This experiment was twice repeated and the same result obtained.

Evidently, then, *Bacillus phosphorescens* is capable of so adapting itself as to produce light at a higher temperature than the normal maximum for light production. This new race thus obtained by adaptation to environment was, however, rather delicate. When kept at a temperature above the growth maximum for a few minutes the bacteria did not again emit light, either when brought back to 35° or even to 26,° until they had been previously transferred to fresh media; and then it was a new generation which produced light. Again, exposure to a low temperature showed this new race to be quite weak. After 24 hours exposure to a temperature of 0° it was usually 48 hours after a gradual or sudden change to either 26° or to 35° before light was again produced. I was unable to observe any adaptation above 35°.

These experiments show that neither sudden or gradual changes of temperature within the limits for light production affect the intensity of light. Further, while the bacteria may adapt themselves to higher temperatures and produce light above the normal luminescence maximum, still this is not possible for low temperatures, since the minimum temperatures for growth and luminescence are coincident. It is further to be noted that notwithstanding the greater adaptability for light production at higher temperatures, exposures to temperatures but slightly above the growth maximum are much more injurious than exposure to temperatures much below the growth minimum. Very low temperatures appear to act as a stimulus, since subsequent luminescence is far stronger than in cultures kept continuously at the optimum temperature. This was the only stimulating effect produced by temperature which was observed.

Relations to Illumination.

Only Dubois has noticed any effect of illumination on luminescence. He observed a slight dimunition of light production as a result of continued illumination. My observations do not show such an effect.

Good young luminous cultures were placed at various temperatures between the minimum and maximum for luminescence. These were divided into three lots; one was kept in continued darkness, another in alternate light of day and darkness of night, and still another exposed continuously to a 16 candle power incandescent light placed 2 feet away. These three sets of cultures were kept under observation for 48 hours. At the end of that time all were luminescent and there was no evident difference in the intensity of the light of any of the cultures.

Apparently a certain amount of continued illumination is without effect on the power of light production. It is, however, not only possible, but also probable that *very* strong illumination would not only destroy luminescence, but also the organisms as well.

The Effect of Ether.

One c.c. of ether added to a luminous culture of *Bacillus phosphorescens* at once destroys luminescence. This effect is, however, as much physical as physiological, for the ether spreads as a thin film over the surface of the culture and excludes free oxygen.

To ascertain the physiological effect of the ether, it was used both in water solution and in vapor form. To good luminous bouillon cultures 10% ether water was added in sufficient amount to make 1% ether in the culture. In all such cases the light was at once extinguished. After 2 to 3 hours, however, the cultures were as brightly luminous as ever. When 5% ether water is added in sufficient amount to have .5% ether in the culture, luminescence does not cease or only after from 30 to 45 minutes and then the culture rarely remains dark for about an hour. The light return is in all probability due to the evaporation of the ether. While .5% of ether in the culture may then at times cause narcosis, as much as 1% is to be considered as about the minimum amount needed to regularly produce narcosis.

In order to determine whether all or only some of the activities of the organism were held in abeyance, the effect of the prolonged action of ether was investigated. To good luminous cultures 5% ether water was added in sufficient amount to make .5% ether in the culture. The culture thus treated was placed together with an open dish of 5% ether water under a large bell-jar. The size of the bell-glass insured a sufficient quantity of free oxygen and at the same time retained the ether vapor. In the course of 15 to 35 minutes light was no longer evident in the cultures and they remained dark while under observation which lasted, in some cases 3 days, in others 1 week. The growth in the cultures was meanwhile luxuriant. It is noteworthy however, that a surface film was rarely formed and that the growth was quite evenly distributed throughout the liquid medium. Further the red discoloration and consequent rich production of the lower fatty acids, appeared much later than in untreated cultures. Usually the red color and the fatty acids appeared in from 4 to 5 days after inoculation. In the cultures thus treated with ether this condition did not appear until 1 week or 10 days after inoculation.

Since the photobacteria showed themselves capable of some adaptation to high temperatures, the thought occured that perhaps there might be a similar adaptability to ether. From cultures which had been exposed to the effects of ether as above described for 24 hours, transfers were made to fresh media (*B*) and to these *B* cultures .5% ether added and the daughter cultures placed with the parents under the bell-glass together with the open dish of ether water. The growth in the *B* cultures was luxuriant but no light was produced. After 24 hours growth of the B cultures under ether influence, transfers were made to a third set (*C*) of media. The growth in *C* cultures was good and after 24 hours the cultures were markedly luminescent. Transfers were made to a further set (*D*) of media and these too in 24 hours exhibited not only luxuriant

growth but a strong luminescence. It is to be noted that at every transfer—every 24 hours—the bell-glass was removed a few minutes. When the bell-glass was replaced, a dish of freshly prepared 5% ether water was placed under it instead of the old dish of ether water. In this way the supply of free oxygen was maintained as well as the action of the ether; the amount of ether which could evaporate from a 5% water solution being a limited amount and not enough to exclude the oxygen from the bell-glass and the organisms.

This experiment was twice repeated and essentially the same results were obtained. In one case the *B* cultures when about 4 days old also emitted light, i. e., about 2 days after their daughter (*C*) cultures were luminous.

From these experiments it is clear that ether, when not too concentrated, exerts a partial narcosis on the bacteria. While it inhibits light production, it does not inhibit growth and multiplication and hence not all of the metabolic activities. In the case of ether we find a second adaptation of the organisms to environment.

Nutrition.

Naturally the first culture medium used for the culture of photobacteria was fish—the substratum from which they had been first isolated. Later the organisms were grown on agar and gelatine containing sea salt, peptone, asparagin, etc. The culture media best suited to cultivation of photobacteria are those first used by Beijerinck. These are described in a previous section of this paper as normal fish bouillon, fish agar and fish gelatine.

That nutrient conditions exert some effect on light production, we know from Beijerinck's researches. His results indicate that certain substances which are plastic are not photogenic and vice versa.

Further it seems for the six species studied by Beijerinck—which include those I have examined—part of the nitrogen must be furnished as peptone and in some cases all of the nitrogen may be given in this form. In some species (peptone forms) peptone will alone cover all the carbon and nitrogen requirements, while in other species (peptone-carbon forms), although peptone will suffice for the nitrogen needs, an additional source is needed for the carbon. *Bacillus phosphorescens* and *Microspira luminosa* are peptone forms, while *Bacterium phosphorescens* is a peptone-carbon form. In all cases, however, peptone seems to be a necessary part of the nutrient media.

ORGANIC NEEDS.

In order to test the conclusion of Beijerinck that peptone or a related protein was absolutely essential, a large series protein-free media were made up. These were inoculated with *Bacillus phosphorescens* and were kept under observation for from 4 days to one

week. Aside from the various protein-free media of Cohn, Fraenkel, Gamaleia, Nägeli, Pasteur, Proskauer and Beck, and Uschinski, a large number of original synthetic media, free from protein, were devised. In almost all instances negative results were obtained, and hence it will be needless to detail all of the synthetic protein-free media employed.

In the following two media, growth was at times, although not always, obtained:

I.	II.
Protogen, 1%.	Protogen, 1%.
$NaNO_3$, 1%.	Glycerol, 1%.
in distilled water.	Glucose, 1%.
	$NaNO_3$, 1%.
	in distilled water.

In both of these media growth was slight and after 3 or 4 days entirely ceased. In no case, however, was any luminescence evident. The growth in medium I. was better than that in II. Protogen which formed the basis of these media is, however, a complex substance, the composition of which is hardly understood, and it may prove to be a protein compound.

While media containing peptone and needed inorganic salts will permit growth and luminescence, still the addition of certain amides causes a more luxuriant growth. These amides include asparagin, lactamid, isobutylamin, isovaleramid, and glycocoll. Asparagin is considered by Beijerinck to be specially stimulating to luminescence. In my experience, while it very much promoted growth, it did not cause the least increase in the intensity of the light produced. Leucin, tyrosin, and sodium asparaginate were apparently without effect. On the other hand, methylamin, hexamethylamin, hexamethyltetramin, uric acid, hippuric acid and alanin, all nitrogen containing compounds, were injurious since they either retarded growth and light production or entirely prevented growth.

The inorganic nitrogen compounds as a rule did not prove plastic or photogenic. Among ammonia compounds, only the valerianate accelerated growth; it did not, however, affect the light intensity. The following ammonium compounds proved either injurious or at least indifferent:—tartrate, bimalate, chloride, carbonate, nitrate, sulphate, phosphates, and aldehyde-ammonia.

Nitrate of sodium proved not alone plastic but particularly photogenic. The nitrates of potassium, lithium and calcium proved neither plastic nor photogenic.

In none of the synthetic media containing peptone, amides and inorganic salts, in which distilled water was used as the solvent, was the growth nearly as good as when, in place of the distilled water, fish extract was used as the solvent for the peptone, amides and inorganic salts. Evidently, while peptone may be an essential organic constituent of the nutrient media, it alone or with any of the plastic nitrogen com-

pounds mentioned is not sufficient to produce the best growth of the organisms.

In order to determine whether perhaps a second form of carbon supply was needed, a series of sugars and related compounds were added to the media as secondary sources of carbon. When, in addition to peptone, 1% of either dextrose, lactose, cane sugar or dulcite, was added, the growth was a little more than that in the control without the sugar or the alcohol. Maltose (1%) at first accelerated growth, then retarded it, and later again caused acceleration. Arabinose and levulose retarded both growth and luminescence, while the presence of 1% of inulin was sufficient to entirely prevent growth. The glucosides arbutin, æsculin and agaracin retarded growth or were at least indifferent in their action. A number of additional organic compounds were experimented with. Among these protogen, lecithin, glycerol, sodium lactate, sodium phospholactate and sodium oleinate produced increased growth, but seemed without effect on light production. The following retarded growth: ethyl alcohol, butter, palmitin, stearin, cholestrin, camphor, turpentine, xylol, and citrus, olive, and bone oils.

Peptone and sea-salts dissolved in distilled water constituted a medium sufficient to enable the photobacteria to produce fully as intense a light as when fish or fish extract, peptone and sea salt, etc., were employed. The growth was, however, never as luxuriant in the purely synthetic media as in the media containing fish extract.

MINERAL NEEDS.

All observers have emphasized the fact that in order to insure the best growth a certain amount of sea salt must be added to the culture media. Beyond this nothing is known concerning the inorganic needs of the photobacteria. One great difficulty in the way of investigating mineral needs of luminous bacteria is the fact that peptone must form part of the nutrient medium. All preparations of peptone contain a considerable amount of ash. Grübler's purified peptone, which was the best at my disposal, contained about 1% of ash, while the Witte peptone, which was rarely employed, contains rather more than 1%. The ash of the Grübler peptone (that used in the following experiments) contains iron, barium, sodium and potassium. Since peptone is essential, it at first seemed very improbable that anything could be learned about the mineral needs of the bacteria.

The various culture media employed for preceding experiments have all contained a varying number of inorganic salts. The question then arose, might there not be enough mineral matter in the peptone to provide for its inorganic needs? Distilled water containing 1 or 2% of peptone, however, remained free from growth even a week after inoculation. When, however, 2% of sea salts was added to the 1% peptone water fairly good growth and a very strong luminescence were obtained. In order to learn whether the complete mixture of salts contained in sea water was necessary, or only certain of these, media were made up con-

taining, in addition to 1% peptone, varying quantities of each of the salts in sea water.

Since NaCl constitutes the bulk of the sea salt, it was first experimented with. To a series of flasks containing 1% peptone in double distilled water, NaCl was added in amounts of .25, .5, 1, 2, 3, 5, 10 and 15 per cent, respectively. Each of these flasks of peptone and NaCl was inoculated with *Bacillus phosphorescens*. After 18 hours, growth was present in all of the media except the one containing .25% of NaCl. At no time within the course of the next ten days was any growth whatever to be found in this flask. The growth in the flasks containing .5, 10 and 15% of NaCl was very slight, that in the latter two being less than in the first. The growth continued in the cultures with 10% and 15% of NaCl for only a few days and then ceased entirely. Cultures containing 1%, 2% and 3% NaCl showed a luxuriant growth, which continued for nearly a week. In the cultures containing .5% and 5% NaCl, growth continued for 9 or 10 days.

While growth occurred in all except one of these peptone NaCl media, in only three was any luminescence to be observed. The cultures containing 1%, 2% and 3% NaCl all emitted a strong light. In none of the other cultures was light discernable at any time. The light in these three cultures was fully as bright as when the bacteria were grown in normal fish bouillon. The growth was not, however, quite as luxuriant. These experiments were repeated five times and yielded the same results.

Evidently, then, a single one of the ingredients of sea salt (NaCl) is sufficient for the needs of luminescence. Would any one of the other salts contained in sea water or belonging to the groups of alkali or alkaline earth metals do just as well as NaCl? To answer this question, to 1% peptone in distilled water I added the following amounts of $MgCl_2$: .1%, .25%, .5%, 1%, 2%, and 5%, respectively. In 18 hours, growth was evident in all of these except the one containing but .1% $MgCl_2$. Only those cultures containing 1% and 2% $MgCl_2$, however, became luminous. The light in these cultures appeared from 24 to 48 hours later than in the corresponding NaCl cultures and was rather weak.

Media in which KCl, $CaCl_2$, NH_4Cl, and $BaCl_2$ were employed in the place of NaCl remained perfectly clear for a week after inoculation. In addition to the above salts, the following were also used in the place of NaCl; KNO_3, K_2SO_4, $LiNo_3$, $RbSO_4$, $Ca(NO_3)_2$, and $Sr(NO_3)_2$. None of these were, however, even sufficient for growth of the organism—the media remaining perfectly clear for the week during which they were observed.

Two of the salts of sea water, NaCl and $MgCl_2$, are evidently of prime importance for the growth and light production of these bacteria, and are interchangeable. Further, the optimum amount of $MgCl_2$ approximates the optimum amount of NaCl. The remaining salts of sea-water are insufficient for the needs of the photobacteria. Not only is this so, but the addition of potassium or calcium salts to a peptone-NaCl medium appears to retard the growth and also light production.

The question then presented itself as to whether the metal or the haloid was the important element, or whether both were required. If other salts of sodium could replace the chloride, then the metal would be the important element. If this were not the case, then the chlorine ion or the entire molecule would be required. To test this I used $NaNO_3$ in the place of NaCl. Not only did I get growth in such media, but the light obtained with 1%, 2%, and 3% of the salt was even more intense than when the chloride had been used. Further, the minimum amount of $NaNO_3$, like that of NaCl, was .5%. Less than this was not sufficient for growth.

The sulphate of sodium, Na_2SO_4, was likewise found capable of replacing NaCl, and while the growth was fully as good as when NaCl was used, still the cultures did not become luminous as soon, nor were they as bright as when NaCl was used.

In addition to the chloride, the nitrate and the sulphate of sodium, ten other sodium salts were experimented with, namely:—monobasic phosphate, dibasic phosphate, sulphite, phospho-lactate, citrate, carbonate, acid carbonate, nitrite, tartrate, and bitartrate. Of these the first five when added to a 1% peptone solution were sufficient for growth, and, except the sulphite, were sufficient for luminescence. The remaining five salts were found not to be able to replace NaCl. This insufficiency is, however, in all probability, due to the character of the ion linked with the sodium rather than to the sodium ion itself, since eight of the thirteen salts of sodium investigated, when added in sufficient quantity to 1% peptone solution gave good growth.

Since $MgCl_2$ was capable of replacing NaCl, it seemed strange that the closely related KCl could not replace NaCl. In order to make sure that the insufficiency of KCl was not an osmetic one, media was made in which KCl and KNO_3 were added to peptone in quantities isoosmotic with 1, 2 and 3% of NaCl and $NaNo_3$, respectively. However, in no case was any growth to be observed even a week after inoculation.

Of the salts of sodium, the nitrate, chloride and sulphate are the best forms in which to furnish sodium to the bacteria. When the nitrate is used, a far brighter light is obtained than when any of the other salts are employed. The chloride is also used to better advantage than is the sulphate. The fact that the nitrate is more advantageous than the chloride of sodium indicates that the sodium need can hardly be a question simply of adaptation to its primitive invironment.

The Theory of Luminescence.

At present, those who have studied the luminous bacteria may fairly be said to be divided into two camps, one holding that luminescence is intracellular and that it is inseparably bound up with life, while the other considers it to be extracellular, and not inseparable from life—that it is capable of reproduction in the laboratory. Among those holding the intracellular view may be mentioned Pflüger, Beijerinck and Lehmann.

Radziszewski, Ludwig and Dubois believe in the extracellular theory. The observations at hand give almost equal support to both sides of the question, although the intracellular theory seems to have a little the better of the argument.

That light is an oxidation phenomenon, is pretty largely accepted by all students of the luminous bacteria. The questions to be settled are, however, numerous, e. g., what it is that is oxidized, the conditions of oxidation, how the light is produced by oxidation, and is the oxidation internal or external? We know that even in an abundant supply of fresh oxygen the photobacteria may be non-luminous. Further, continued growth and light production do not necessarily go hand in hand. We have seen that photogenic bacteria may grow at a high temperature without producing light, e. g. *Bacillus phosphorescens* will grow at 38°, but remains perfectly dark.

A culture of *Bacillus phosphorescens* does not emit light as soon as the first growth takes place. Usually it is not luminous until from 18 to 24 hours after inoculation. During this period the culture medium is seen to become more and more clouded with a white growth, and finally a white skin of bacterial growth covers the surface of the culture liquid. Then the culture becomes luminous. This is not due to contact with the air, because when the culture is luminous, it is luminous to a depth of 2 to 3 centimeters. Again we have seen how a certain amount of ether may prevent light production and yet not growth.

During the 18 hours immediately following inoculation, and before luminescence begins, the bacteria are actively swimming about the culture liquid. After light production begins not only are the bacteria of the surface skin motionless, but also those in the depths of the liquid. In no case have I observed light while the bacteria were motile, and conversely I have not been able to find the bacteria in motile condition while they were in a luminous condition. Indeed it would seem—at least for *Bacillus phosphorescens*—that light and motion are opposing functions, since they are not performed at the same time, but one follows the other.

As long as the bacteria are in motion, the culture has but little odor (unless fish extract has been used) and is of a light yellow color. Shortly after the culture becomes luminous, the color changes. First it becomes dark yellow, then it is light brown, then more and more reddish. By this time the odor is very marked and reagents are hardly needed to demonstrate the presence of the lower fatty acids and of skatole. And reagents confirm the olfactory evidence.

It has been shown that the elements sodium and magnesium are of importance for both light and growth of luminous bacteria. Just what the connection may be between the oxidation which causes light emission and sodium or magnesium is still a question needing further experimentation. That there is some connection is clear. A comparison of luminous bacteria with the electric ray is rather suggestive. The electric organ of the torpedo is known to be rich in NaCl, usually having as much as 3%. Further, it is commonly accepted that the electric organ

is modified muscular or contractile tissue. In any case there is analogy between the bacterial contractile flagellæ and contractile animal muscle. Today physicists are pointing out the close connection between light and electricity. The large NaCl content in the electric (or modified contractile) organ of the torpedo on the one hand and the large sodium need of the photobacteria taken with the relations between light and motion on the other hand are full of significance.

With the facts at hand one may reasonably draw a few conclusions concerning the nature of light production. The fact that no luminous substance has ever been certainly isolated rather inclines one to disbelieve the extracellular theory. The fact that the temperature limits for life are without the limits for luminescence points to the intracellular theory. The fact that a slight amount of ether may cause a cessation of light emission and yet not stop growth points in the same direction.

While these facts lead us to strongly believe that luminescence is an internal (oxidation) process, yet there are not facts enough at hand to warrant the assumption that this process is inseparable from life and incapable of exact reproduction in the laboratory. The beautiful researches of Radziszewski show us the possibility of such a thing. Still it is yet to be proven that these same processes occur in the bacterium and are responsible for its luminescence. I see no warrant for Beijerinck's assumption that light is produced by sudden union of oxygen and peptone at the moment of conversion into living protoplasm. To begin with, it is still to be demonstrated that peptone is capable of *direct* conversion into protoplasm. Snythesis is not always a recapitulation of analysis.

To me it seems that luminescence is connected with metabolism, and since its appearance is closely followed by the presence in the culture liquid of the products of portein decomposition, that it is a phase of destructive metabolism. It also seems highly probable that the phenomenon of contractility (motility) and luminescence are closely related to one another, since the one appears when the other disappears. Further, it seems possible that the sodium ion may serve as a strongly reducing agent, possibly rendering oxygen atomic and so providing for a very active oxidation with consequent liberation of energy as light.

In the near future I expect to be able to test the hypothesis suggested in the latter part of this paper.

Summary.

In conclusion I may summarize the chief results of the experiments as follows:

1. All acids are injurious to light production. A slight excess of alkali is even more injurious than a slight excess of an acid.

2. The temperature limits for light emission are *within* those necessary for growth.

3. Change of temperature, either sudden or gradual, is without effect on luminescence, i. e., does not stimulate.

4. There is no luminescence at or below 0°.

5. Exposures to temperatures above the growth maximum are highly injurious to the power of light production, while exposure to very low temperatures seems to serve as a stimulus to light production.

6. *Bacillus phosphorescens* is capable of adapting itself to high temperatures, producing a race capable of light production at 35°, which is 5° above the normal maximum for luminescence.

7. A certain degree of continued illumination is without effect, and it is possible for the bacteria to live their entire lives in the dark and yet emit a brilliant light.

8. Ether acts as a narcotic, preventing luminescence, but not growth and multiplication.

9. It is possible to develop a race of bacteria so immune to the action of small amounts of ether as to be still luminous in its presence.

10. Peptone or related protein is required for the nutrition of luminous bacteria.

11. Dextrose, and certain of the higher sugars may be utilized advantageously by *Bacillus phosphorescens.*

12. Either sodium or magnesium is required for growth, and especially for light production. Minimum, maximum and optimum amounts of sodium are observed for growth and luminescence.

13. Potassium, ammonium, lithium, rubidium, calcium, barium and strontium cannot replace sodium (or magnesium).

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